Cholesterol-Fed and Casein-Fed Rabbit Models of Atherosclerosis
Part 2: Differing Morphological Severity of Atherogenesis Despite Matched Plasma Cholesterol Levels

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Abstract One-month-old male New Zealand White rabbits were fed either a cholesterol-free casein diet (n=10) or low-level cholesterol-supplemented chow (n=10) for 24 weeks, during which total plasma cholesterol levels were matched. After perfusion fixation, aortic tissue samples were taken from six predetermined locations and embedded in epoxy resin for examination by light and electron microscopy. Frozen sections were also obtained for histochemical demonstration of collagen and elastin. Lesion morphology was classified in toluidine blue-stained, semithin epoxy sections as early fatty streaks (round foam cells with little intervening extracellular matrix); advanced fatty streaks (foam cells with extracellular lipid); fibrous plaques (spindle-shaped cells within extracellular matrix); or atheromatous lesions (presence of an atheromatous core). In representative specimens, electron microscopy showed that the ultrastructure of round foam cells was consistent with macrophage derivation, whereas most spindle-shaped cells were clearly smooth muscle cells. Fibrous plaques were more common in the distal than the proximal aorta. Lesions in the casein-fed animals were essentially equally distributed among the four morphological categories, whereas lesions in the cholesterol-fed rabbits were predominantly of the atheromatous type. Thus, cholesterol-fed rabbits had, in general, more advanced lesions than casein-fed rabbits with matched total plasma cholesterol levels. Moreover, the feeding of a low-level cholesterol diet (0.125% to 0.5% by weight) to rabbits for a relatively short time (6 months) led to the development of advanced lesions similar to those seen in humans. (Arterioscler Thromb. 1994;14:105-114.)

Key Words • rabbits • casein • cholesterol • lesion formation • hypercholesterolemia • atherosclerosis • aorta • OTAP

Traditionally, the cholesterol-fed rabbit has been used as a model of human atherosclerosis because it allows for the rapid development of aortic lesions. Rapid lesion development has been achieved with high levels of dietary cholesterol (2% to 4% by weight), which has resulted in exceedingly high total plasma cholesterol (TPC) levels and in lesions morphologically dissimilar to those seen in humans.1-5 But humans neither ingest large quantities of cholesterol nor normally have TPC levels exceeding 1000 mg/dL. Morphologically, human atherosclerotic plaques consist primarily of smooth muscle cells (SMCs) and contain a fibromuscular cap covering a core composed of extracellular lipid and necrotic debris.3,6-9 The intimal SMCs synthesize collagen and elastin, which contribute to the fibrous nature of the atherosclerotic plaque.3,9,10

Various methods have been used to induce human-type lesions in rabbits, including intermittent cholesterol feeding,11 the use of semisynthetic diets containing casein12-14 or casein and cholesterol,15,16 and the injection of foreign proteins.17 Despite the fact that researchers have reported human-type lesions in rabbits, the rabbit is still identified as a less suitable model for the study of atherosclerosis than swine5,9,18 and nonhuman primate models.3,5,9 In fact, some reviews continue to cite rabbits as having lesions that consist primarily or entirely of macrophage-derived foam cells.1,3,5

More recently, researchers have found that simply reducing the level of dietary cholesterol (0.2% to 2% by weight) given to New Zealand White (NZW)19-21 ChinChilla,22 and Dutch Belted rabbits23-24 results in more human-type lesions. These lesions contain the large numbers of SMCs,19-21,23 extracellular matrix deposition,19,21,23,24 and cholesterol crystals (clefts)22,24 that are typical of more human-type lesions.

Given the controversy over the type of lesion formation observed in the rabbit, the experiments described in this article were designed to determine if NZW rabbits fed either a semisynthetic diet enriched in casein or a low-level cholesterol diet (0.125% to 0.5% by weight), with comparably lower TPC levels than have been usually induced in rabbits, develop human-type lesions. The results of both light and electron microscopic examination of lesion formation are presented.

Methods

Aortic tissue, perfused under pressure with dilute Karnovsky's fixative,25 was obtained from animals as described in the accompanying article.25 Briefly, the experimental groups consisted of animals fed a casein-enriched diet (CAS; n=10) and animals fed cholesterol-supplemented (0.125% to 0.5%) rabbit chow (CH; n=10). Three control animals fed standard rabbit chow were also examined morphologically.
Tissue Preparation

Routine Plastic-Embedded Sections

Once aortas had been removed from the animals and cleaned of the bulk of adhering fat, the vessels were divided into the arch, the proximal descending thoracic aorta, the distal descending thoracic aorta (just proximal to the celiac artery), and the abdominal aorta (just proximal to the iliac bifurcation). Samples for plastic embedding were systematically taken from the ascending aorta, the arch, the region distal to the first pair of intercostal ostia, the ventral wall opposite the first pair of intercostal ostia, the abdominal aorta just distal to the celiac artery, and the abdominal aorta just distal to the first pair of lumbar arteries.

Samples were washed in 0.06 mol/L phosphate buffer (pH 7.6), postfixed in 1% OsO$_4$, for 1 hour on ice, washed again in 0.06 mol/L phosphate buffer, dehydrated in a series of alcohols, and cleared in propylene oxide. Samples were then infiltrated with 50% propylene oxide:50% Araldite/Polybed 812 embedding medium (Polysciences Inc, Warrington, Pa) for 12 hours on a rotator followed by 100% Araldite/Polybed 812 for 8 hours. Samples were then embedded in Araldite/Polybed 812 in flat molds at 60°C for 48 hours.

Semithin sections (1 μm) were cut for light microscopy on a Sorvall Porter-Blum ultramicrotome with glass knives, stained with 0.1% toluidine blue in 1% sodium borohydride for 4 minutes, and then rinsed with distilled water. Slides were dried in a 60°C oven overnight and then coveredslipped with Araldite/Polybed 812 as a mounting medium. Ultrathin sections were cut at 70 to 90 nm using an LKB Ultrotome III and were stained with 7% uranyl acetate in 50% ethanol followed by Sato’s lead citrate.\textsuperscript{27}

OTAP Plastic-Embedded Sections

Tissue specimens from six rabbits in each experimental group (CAS and CH) were taken from the region of the abdominal aorta proximal to the celiac artery. Samples were rinsed twice in 0.1 mol/L sodium cacodylate buffer (pH 7.4) and then processed for electron microscopy by using the osmium–tannic acid–paraphenylenediamine technique (OTAP; previously abbreviated TA-PDA), which enhances the preservation and identification of atherosclerotic lipid deposits in humans and rabbits.\textsuperscript{28} Briefly, the OTAP procedure involved an initial treatment of the tissue with cacodylate-buffered 2% OsO$_4$, and extensive washing in the same buffer, followed by mordanting with 1% buffered tannic acid (Fisher Scientific, Pittsburgh, Pa) and a wash in 1% Na$_2$SO$_4$. During the initial stages of dehydration, tissues were exposed to 1% paraphenylenediamine (Sigma) in 70% ethanol. The dehydration schedule then proceeded through 70%, 95%, and 100% ethanol within 45 minutes to a 1:1 mixture of LX112 resin (Ladd Research Industries, Burlington, Vt) and ethanol for 1 hour, followed by pure resin overnight. On the following day, two changes of LX112 resin were made, and then the tissue was embedded in LX112. Ultrathin sectioning and staining were performed as described above.

Frozen Sections

Each segment of the thoracic aortas was embedded in OCT Compound (Miles Inc, Elkhart, III) and frozen in liquid nitrogen as described in the accompanying article.\textsuperscript{29} Longitudinal frozen sections (15 μm thick) were cut along the full length of the dorsal aspect of the thoracic aorta with a Leitz 1720 digital cryostat. The sections were then stained with aldehyde fuchsin and picrosiris red for elastin and collagen, respectively,\textsuperscript{29} dehydrated in a graded series of alcohols, and mounted in a resinous mounting medium.

Light Microscopy and Lesion Classification

Toluidine blue–stained plastic sections and aldehyde fuchsin/picrosiris red–stained frozen sections were examined and photographed on a Zeiss Axioshot microscope with Kodak technical pan 2415 (black and white film) and Kodak Ektar 25 (color film), respectively. The toluidine blue–stained plastic sections were used to determine lesion composition. The aldehyde fuchsin/picrosiris red–stained frozen sections were used to examine the pattern of collagen and elastin staining in the lesions.

Plastic-embedded sections from each area of the aortas outlined above were used to classify lesions according to a system (Table 1) that combined various aspects of classification systems used by other groups that have examined lesions in rabbits,\textsuperscript{20,30-31} nonhuman primates,\textsuperscript{32} and humans.\textsuperscript{7,8,33-35} Slides were coded so that the examiner did not know which dietary group was represented in each case. Electron microscopy was used to examine selected lesions at the ultrastructural level and confirm the identity of the different cell types found.

We defined early fatty streaks, as others have,\textsuperscript{7,20,30,34,36} as lesions that have predominantly rounded foam cells that, by electron microscopy, appear to be macrophage derived. These lesions are similar to Stary’s type I lesions and his type II fatty streak.\textsuperscript{8} Our advanced fatty streak resembles McGill’s progressing fatty streak\textsuperscript{38} and Masuda and Ross’s fibrofatty lesion\textsuperscript{39} in that it has both lipid-filled macrophages and lipid-filled SMCs along with the extracellular lipid characteristic of Stary’s type III preatheroma. The fibrous plaque described here is unlike that in other classification systems\textsuperscript{8,12,35} because it consists primarily of large numbers of smooth muscle–like cells without an identifiable
FK3 1. Photomicrographs of diet-induced aortic atherosclerotic lesions showing the range of morphologies seen in both casein- and cholesterol-fed rabbits. The internal elastic lamina is marked in the bottom left of each panel (arrow). A, Lesion from the abdominal aorta (just distal to the celiac artery) of a casein-fed animal showing the classic morphology of a rabbit fatty streak and containing stacks of large round foam cells. B, A typical advanced fatty streak from the arch of a cholesterol-fed rabbit that contains approximately equal areas filled with round foam cells (arrowheads) and non-lipid-filled cells near the luminal aspect of the lesion; some of the latter are spindle shaped (arrows). C, A fibrous lesion from the ostium near the first pair of intercostal arteries of a cholesterol-fed rabbit that consists predominantly of spindle-shaped cells and extracellular matrix that span the thickness of the lesion; a few lipid-filled cells (arrowhead) are also seen. D, An atheromatous lesion from the region near the first pair of intercostal ostia of a cholesterol-fed rabbit that has a developing core near the internal elastic lamina containing pools of extracellular lipid, necrotic debris, and cholesterol crystals, overlaid with a fibrofatty cap. Bar=50 μm.

atheromatous core. Our atheromatous lesion is characterized by a variably well-developed fibrous cap overlying a necrotic core of cellular and extracellular debris that is similar to Star's type IV atheroma and Haust and More's atheromatous lesion.

Electron Microscopy
Routine and OTAP plastic-embedded sections were examined by electron microscopy by using a JOEL 200CX microscope operating at 80 kV. Routine sections were used to examine lesion composition to characterize both cellular and extracellular constituents. OTAP sections were used primarily to identify and characterize the lipid-containing elements of these lesions.

Results
Lesion Morphology
Plastic-embedded, toluidine blue-stained semithin sections were classified under the light microscope, without knowledge of the dietary group, according to the system outlined in Table 1. Sections with identifiable lesions were examined and categorized as early fatty streaks, advanced fatty streaks, fibrous plaques, or atheromatous lesions.

Most of the samples from both experimental groups had microscopically detectable lesions, with varying morphologies seen in both groups (Fig 1). Atherosclerotic lesions were not detected in control animals. Thin, early fatty streaks consisted of what appeared to be isolated macrophage-derived foam cells, and thicker fatty streaks (Fig 1A) consisted of stacks of these lipid-filled cells. At the electron microscopic level, the early fatty streak lesion was confirmed as being densely packed with large, round, lipid-laden cells but little extracellular matrix (Fig 2A). Near the luminal surface and extending to the midintima, these rounded cells almost always lacked a surrounding basement membrane and generally possessed micropodia typical of macrophage-derived foam cells. In the deep intima, near the internal elastic lamina, lipid-laden SMCs and extracellular lipid deposits, including cholesterol clefts, were found. In samples prepared using the lipid-preserving OTAP technique, lysosomal lipid inclusions became more evident alongside the more easily recognized cytoplasmic lipid droplets (Fig 2B).
Advanced fatty streaks consisted of approximately equal numbers of macrophage-derived foam cells and SMC-derived foam cells, with the amount of extracellular matrix and extracellular lipid varying from lesion to lesion (Fig 1B). At the electron microscopic level, both macrophage-derived cells, lacking a basement membrane and possessing numerous lysosomes, and SMC-derived cells, some containing myofilaments and others containing an abundance of rough endoplasmic reticulum, were present with variable amounts of intracellular lipid (Fig 3).

Lesions identified as fibrous plaques consisted predominantly of spindle-shaped cells, extracellular matrix, and variable amounts of extracellular lipid (Fig 1C). When examined with the electron microscope, the fibrous lesions were found to be composed predominantly of SMCs of the contractile phenotype, characterized by sparse rough endoplasmic reticulum but with an abundance of myofilaments in the cytoplasm (Fig 4).

Lesions identified as atheromatous were characterized by either a developing or well-developed necrotic core filled with debris and what were once pools of extracellular lipid and cholesterol crystals (Fig 1D). In addition, these lesions usually had a well-developed fibrous cap. By electron microscopy, the necrotic core of the atheromatous lesion contained cellular debris and membranous whorls indicative of extracellular lipid deposits as well as cholesterol clefts (Fig 5). Because cholesterol clefts could be identified in each of the four lesion types, their presence was not used as a differentiating factor in the categorization of lesions.

Electron micrographs of the more advanced atheromatous lesions preserved by the OTAP procedure revealed the presence of intracellular neutral lipid as well as extracellular neutral lipid and lipid vesicles (Fig 6). Cholesterol clefts were often found associated with vesicular lipid deposits (Fig 7). No significant morphological differences relating to diet (CAS versus CH) were found when OTAP-prepared tissues were examined by electron microscopy.

Frozen sections stained with aldehyde fuchsin and picrosirius red confirmed the presence of elastin and collagen, respectively, in lesions of both the CAS and CH groups. The most notable feature was the varying amount of collagen seen in different areas of these longitudinal sections. Areas that contained very little collagen in the intima (Fig 8A) likely corresponded with areas in plastic sections that were filled with macrophage-derived foam cells. Other areas contained relatively more collagen (Fig 8B), while still others contained thick parallel bands of collagenous material near the luminal aspect of the lesion (Fig 8C) that probably corresponded with lesions (fibrous or atheromatous) containing a thick fibrous cap. Rela-
tively less elastin than collagen was seen in the intima. Intimal elastin usually appeared as a fine meshwork of fibers (Fig 8B).

Lesion Classification and Distribution

Lesions from the CAS and CH dietary groups were classified according to lesion type for each area of the aorta sampled. The percentage of sections showing each type of lesion is outlined in Table 2.

Lesions of each of the four types were seen in both groups. Examination of the regional distribution of lesions in the CAS and CH groups showed that fibrous plaques, which comprised 33% of lesions in two abdominal aortic sites combined, were much less common in

Fig 3. Electron photomicrograph of an advanced fatty streak from the abdominal aorta of a cholesterol-fed rabbit. Lipid droplets are found both in smooth muscle cells, with myofilaments and surrounding basement membrane (arrow), and in cells lacking these features, presumably macrophages (asterisk). At the top left, the endothelial monolayer is breached, and the foam cell is in direct contact with the lumen. Bar=2 \( \mu \)m.

Fig 4. Left, Electron photomicrograph of a fibrous lesion from the ventral wall in a cholesterol-fed rabbit. The lesion consists almost entirely of smooth muscle cells of the contractile phenotype with abundant myofilaments. Note that the cells are surrounded by a dense matrix of fibrillar collagen and reticular basement membrane. Abundant small cholesterol clefts (arrowheads) and pools of extracellular lipid (arrows) were found in the extracellular matrix. Photomicrograph was obtained from the same block of tissue as Fig 1C. Bar=1.5 \( \mu \)m. Inset, Increased reticular basement membrane (asterisks) adjacent to smooth muscle cell. Bar=1 \( \mu \)m.
the aortic arch. When sections were analyzed from the aorta as a unit, it was evident that approximately equal numbers of lesions in the CAS group could be categorized as early fatty streaks (20%), advanced fatty streaks (23%), fibrous lesions (25%), or atheromatous type lesions (32%), whereas lesions in the CH group were predominantly of the atheromatous type (61%). Interestingly, in both groups the early fatty streak comprised the smallest percentage of lesions.

Discussion

In this study we used a classification system composed of four categories including the early fatty streak, the advanced fatty streak, fibrous lesions, and atheromatous lesions. This system best describes the range of lesions seen in the CAS and CH groups.

The variable aortic lesion morphologies identified in both groups are very interesting, given the results reported in the literature. Some investigators have reported that casein-fed rabbits develop human-like fibrous lesions, while others have identified only fatty streaks in these animals. In the cholesterol-fed rabbit, researchers have identified either fibrous-type lesions or fatty streaks but usually not both. Others, using a combined casein-cholesterol diet, have identified only fibrous-type lesions in their rabbits. Only two groups appear to have described a range of lesion types, from fatty streaks to advanced fibrous plaques, in their investigation of aortic atherosclerosis in young rabbits. The rabbits in the study by Tsukada et al were fed 0.2% cholesterol/20% casein for up to 13 months, and the rabbits in the study by Wilson et al...
were fed 19% butter/20% casein for 6 to 60 months. Our findings are consistent with both of these studies, in that a range of lesion types was evident in the aortas of hypercholesterolemic rabbits whether they were fed a cholesterol-supplemented or a casein-enriched diet. However, our study seems to be unique in that the entire range of lesions, including advanced atheromatous lesions, could be seen as early as 6 months after the induction of hypercholesterolemia.

Like many investigators, we used electron microscopy to confirm the predominance of macrophage-like foam cells in early fatty streaks and of SMCs in fibrous plaques and atheromatous lesions. Our results are consistent with those of Tsukada et al., who used monoclonal antibodies to identify the variable presence of macrophages and SMCs in the lesions of Watanabe heritable hyperlipidemic and cholesterol/casein-fed NZW rabbits. Using similar criteria to define their four lesion categories, they found the same range of lesions that we found in our animals. In addition, the presence of cholesterol crystals was confirmed by electron microscopy in all of our lesion types but was most common in the advanced lesions. The presence of both SMCs and cholesterol crystals is considered to be a common feature of the advanced human atherosclerotic plaque.3,6-9

Few researchers have examined the prevalence of lesion types in the hypercholesterolemic rabbit aorta. One group reported that in young rabbits fed a low level of cholesterol for 18 months, all lesions examined were fatty streaks, while in older hypercholesterolemic rabbits, 78% of the samples were fibroatheromatous and 11% were fatty streaks. They concluded that these advanced lesions were the result of an increased susceptibility of the aged arterial wall to atherosclerotic lesion formation.30,40 In contrast, our study showed that a low-level cholesterol diet given to young rabbits for a period of 6 months resulted in advanced atherosclerotic lesions, with 61% of lesions sampled from the entire aorta of the atheromatous type, 77% of lesions considered advanced plaques (fibrous and atheromatous types combined), and only 12% consisting predominantly of foam cells.

Picrosirius red-stained collagen was conspicuous in lesions from both the CAS and CH groups, confirming the fibrous nature of these lesions. The presence of fibrous intimal components is generally reported in rabbits with diet-induced hypercholesterolemia,12,21,23,24,30 and the synthesis of collagen and elastin is reported to be increased in the aortas of rabbits fed cholesterol- and fat-supplemented diets when the incorporation of 14C-labeled proline into collagen is examined in vitro or in vivo.43 Some of our samples showed a pattern in which bands of collagen-containing lacunae were seen in the luminal aspect of the lesion. This pattern is strikingly similar to that demonstrated by Shekhonin et al.,44 in which fibrous caps containing thick bundles of type I and type III collagen, having similar lacunae-like configurations, are shown in sections taken from the human aorta. In comparison to collagen, lesions in our study contained relatively little elastin.

### Table 2. Prevalence of Lesion Type in the Aorta

<table>
<thead>
<tr>
<th>Location</th>
<th>Early Fatty Streak</th>
<th>Advanced Fatty Streak</th>
<th>Fibrous</th>
<th>Atheromatous</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CAS</td>
<td>CH</td>
<td>CAS</td>
<td>CH</td>
</tr>
<tr>
<td>AS</td>
<td>40%</td>
<td>25%</td>
<td>20%</td>
<td>13%</td>
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<tr>
<td>AR</td>
<td>0</td>
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<td>56%</td>
<td>10%</td>
</tr>
<tr>
<td>VW</td>
<td>40%</td>
<td>17%</td>
<td>20%</td>
<td>17%</td>
</tr>
<tr>
<td>OS</td>
<td>0</td>
<td>11%</td>
<td>20%</td>
<td>11%</td>
</tr>
<tr>
<td>AC</td>
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<td>11%</td>
<td>22%</td>
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<tr>
<td>AL</td>
<td>33%</td>
<td>0</td>
<td>0</td>
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CAS indicates casein-fed rabbits; CH, cholesterol-fed rabbits; AS, ascending aorta; AR, aortic arch; VW, ventral wall at the level of the first pair of intercostal arteries; OS, ostium near the first pair of intercostal arteries; AC, abdominal aorta near the celiac artery; and AL, abdominal aorta near the first pair of lumbar arteries.
that only foam cell–type lesions develop in these animals.

Clearly, the induction of hypercholesterolemia in the rabbit does not result exclusively in lesions containing stacks of lipid-filled cells. In fact, more than 75% of the lesions in our rabbits fed a low-level cholesterol diet were advanced lesions. In this study, extensive morphological examination of lesion formation in rabbits fed a cholesterol diet revealed that, without complicated feeding regimens and without endothelial denudation, advanced atherosclerotic lesions developed in a relatively short time, a finding not previously reported in studies of the cholesterol-fed rabbit.

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